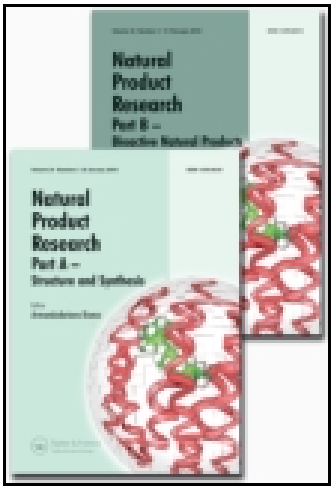

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Microbial metabolism. Part 10: Metabolites of 7,8-dimethoxyflavone and 5-methoxyflavone

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Microbial metabolism. Part 10: Metabolites of 7,8-dimethoxyflavone and 5-methoxyflavone

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Microbial transformation of 7,8-dimethoxyflavone (**1**) by *Mucor ramannianus* produced five metabolites: 7,8-dimethoxy-4′-hydroxyflavone (**2**), 3′,4′-dihydroxy-7,8-dimethoxyflavone (**3**), 7,3′-dihydroxy-8-methoxyflavone (**4**), 7,4′-dihydroxy-8-methoxyflavone (**5**) and 8-methoxy-7,3′,4′-trihydroxyflavone (**6**). It was, however, completely converted to a single metabolite, 7-hydroxy-8-methoxyflavone (**7**) by *Aspergillus flavus*. 5-Methoxyflavone (**8**), when fermented with *Beauveria bassiana*, gave a single product, 5-methoxyflavanone (**9**). Conversion of **8** with *Aspergillus alliaceus* yielded the metabolite 4′-hydroxy-5-methoxyflavone (**10**). The structures of the compounds **2**–**7**, **9** and **10** were established by spectroscopic methods.

Keywords: flavonoids; microbial metabolism; *Mucor ramannianus*; *Aspergillus flavus*

1. Introduction

Flavonoids are polyphenolic compounds found in vegetables, fruits and beverages (Herath, Mikell, Hale, Ferreira, and Khan 2008; Nikolic & van Breemen, 2004; Tasdemir et al., 2006). Cell culture experiments on these compounds show potential use as chemopreventive agents against cardiovascular disease, cancer and other diseases (Middleton, Kandaswami, & Theoharides, 2000). These activities, however, are not often observed *in vivo* in studies of animals and humans due to their very low oral bioavailability (Tsuji, Winn, & Walle, 2006; Walle, Walle, & Halushka, 2001; Walle, Otake, Brubaker, Walle, & Halushka, 2001a; Wen, & Walle, 2006). Excretion facilitated by rapid presystemic hepatic and intestinal glucuronidation and sulphation of the free hydroxyl groups of the flavonoids is responsible for their poor bioavailability (Walle, 2007; Walle et al., 2001a). The observation that methylation of the free hydroxyl groups greatly improves their intestinal absorption and hepatic metabolic stability, limiting metabolism to less efficient CYP-mediated oxidation (Walle, 2007; Wen & Walle, 2006) is the suggested reason for the improvements in some of their biological properties, including the inhibition of cancer cell proliferation (Walle et al., 2007). The early assumption that free hydroxyl groups are necessary for

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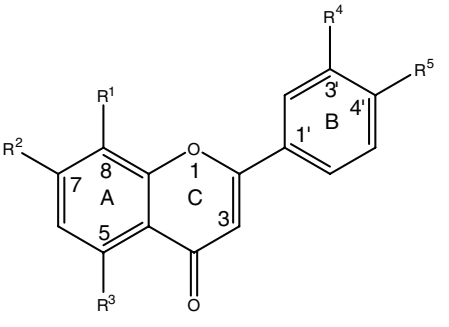
bioactivities of flavonoids (Walle, 2007; Walle et al., 2007) stems from the belief that the main beneficial property of these compounds is their antioxidant activity (Takano et al., 2007; Walle et al., 2007). Lack of such activity in methylated flavonoids has attracted little attention as potent chemopreventive agents, even though many of them occur naturally in a variety of plant species (Wen & Walle, 2006). Some methoxyflavones exhibit multidrug-resistant reversing effects in cancer cells. Their potency is believed to be related to the number and positions of the methoxyl groups (Ohtani et al., 2007). In selecting methoxyflavones as potential chemopreventive agents, it is important to determine how susceptible they are towards metabolism (Walle & Walle 2007). Since microorganisms can be used as predictive models for mammalian drug metabolism, we investigated prospectively the microbial metabolism of 7,8-dimethoxyflavone (**1**) and 5-methoxyflavone (**8**) using 40 microorganisms. Cultures which gave the maximum number of metabolites in good yields were selected for scale-up studies. The structures of the eight metabolites formed (**2–7,9**, **10**) were established by detailed study of their high resolution spectroscopic data.



	R^1	R^2	R^3	R^4	R^5	C-2,3 dihydro
1	OMe	OMe	H	H	H	-
2	OMe	OMe	H	H	OH	-
3	OMe	OMe	H	OH	OH	-
4	OMe	OH	H	OH	H	-
5	OMe	OH	H	H	OH	-
6	OMe	OH	H	OH	OH	-
7	OMe	OH	H	H	H	-
8	H	H	OMe	H	H	-
9	H	H	OMe	H	H	C-2,3 dihydro
10	H	H	OMe	H	OH	-

2. Results and discussion

Microorganisms capable of metabolising **1** and **8** were selected by screening 40 organisms using the standard two stage procedure (Abourashed & Khan, 2000). Scale-up studies of **1** with *Mucor ramannianus* yielded five metabolites: 7,8-dimethoxy-4′-hydroxyflavone (**2**), 3′,4′-dihydroxy-7,8-dimethoxyflavone (**3**) (Menichincheri et al., 2004), 7,3′-dihydroxy-8-methoxyflavone (**4**), 7,4′-dihydroxy-8-methoxyflavone (**5**) and 8-methoxy-7,3′,4′-trihydroxyflavone (**6**). *Aspergillus flavus* converted **1** to 7-hydroxy-8-methoxyflavone (**7**). 5-Methoxyflavone (**8**) yielded a single metabolite each when

fermented with *Beauveria bassiana* and *Aspergillus alliaceus*. They were characterised as 5-methoxyflavanone (**9**) (Lee, Jung, & Jung, 2007) and 4′-hydroxy-5-methoxyflavone (**10**), respectively. The molecular formulae of all the metabolites were determined by HR-ESI-MS.

Metabolite **2** (10 mg, 2.5%) was a white solid with a molecular formula of $C_{17}H_{14}O_5$, corresponding to a monoxygenated product of **1**. Compound **2** showed a hydroxyl band at 3411 cm^{-1} in the IR spectrum. The ^{1}H-NMR data of **2** were similar to those of **1**, except for the B-ring protons, which showed *para*-substitution [δ 7.89 (2H, d, $J = 9.0$ Hz), 6.92 (2H, d, $J = 9.0$ Hz)]. The hydroxyl group was assigned at C-4′ by a detailed NMR study, and the compound was identified as 7,8-dimethoxy-4′-hydroxyflavone.

Compound **3** was obtained as a white solid (30 mg, 7.5%), which gave a molecular ion peak at m/z 313.0750 $[M-H]^+$ in its HR-ESI-MS, corresponding to the elemental formula $C_{17}H_{14}O_6$. The ^{1}H-NMR data of **3** showed close resemblance to those of **2**. The B-ring, however, showed 1, 3, 4 aromatic substitution [δ_H 6.98 (1H, d, $J = 8.5$ Hz), 7.55 (1H, dd, $J = 8.5, 2.0$ Hz), 7.56 (1H, d, $J = 2.0$ Hz)]. It was identified as 3′,4′-dihydroxy-7,8-dimethoxyflavone by correlation spectra. Although **3** had been synthesised previously, limited published data prevented a direct comparison (Menichincheri et al., 2004).

Compound **4** (10 mg, 2.5%) was isolated as a light yellow solid with a molecular formula of $C_{16}H_{12}O_5$. The ^{1}H-NMR data of **4** were also similar to those of **1**, with the exception of the B-ring protons showing *meta*-substitution (Table 1). The ^{13}C-NMR spectrum revealed a highly deshielded singlet carbon at δ 158.4. It was assigned to C-3′ by HMBC correlations. Further, the ^{1}H-NMR of **4** showed that one of the methoxy groups of **1** was demethylated during transformation. HMBC correlations permitted the assignment of the hydroxyl group at C-7. Thus, the structure of compound **4** was established as 7,3′-dihydroxy-8-methoxyflavone.

The NMR data of **5**, a pale yellow solid (37 mg, 9.25%) having a molecular formula $C_{16}H_{12}O_5$, were similar to those of **4** except for the position of the hydroxyl group in the B-ring. It was determined to be at C-4′ by correlation NMR spectra. Compound **5** was thus identified as 7,4′-dihydroxy-8-methoxyflavone.

Compound **6** (7 mg, 1.75%), isolated as a faint yellow solid, was shown to have a molecular formula $C_{16}H_{12}O_6$. Except for the absence of a methyl group, all other NMR resonances of **6** were similar to those of the metabolite **3**. Correlation of the remaining *O*-methyl resonance with the carbon resonance at δ 135.8 (C-8) by HMBC enabled the assignment of structure **6** as 8-methoxy-7,3′,4′-trihydroxyflavone.

Compound **7**, obtained as a white solid (90 mg, 36%), was formulated as $C_{16}H_{12}O_4$. The NMR spectral data of **7** were similar to those of the metabolite **4** except that its B-ring was free of hydroxyl groups. NMR correlation data were used to characterise the compound as 7-hydroxy-8-methoxyflavone.

The white solid **9** (65.4 mg, 7.47%) with a molecular formula $C_{16}H_{14}O_3$ was identified as 5-methoxyflavanone by comparison with published data (Lee et al., 2007).

Metabolite **10**, a white solid (89.9 mg, 71.92% yield) with the molecular formula $C_{16}H_{12}O_4$, corresponded to a monoxygenated product of **8**. A hydroxyl band was observed at 3428 cm^{-1} in the IR spectrum of **10**. The ^{1}H-NMR spectrum of **10** was similar to that of **8** except for the presence of two doublets (AA′ BB′ system, δ 6.93,7.90), indicating hydroxylation at the *para* position of the B-ring. The singlet carbon at δ 161.1 in the ^{13}C-NMR spectrum was assigned to C-4′ by correlation spectra of **10**. Thus, compound **10** was characterised as 4′-hydroxy-5-methoxyflavone.

Table 1. ^{1}H-NMR (600 MHz) chemical shifts of **2**–**7** and **10** (DMSO-d_6).

	2	**3**	**4**	**5**	**6**	**7**	**10**
H-2	–	–	–	–	–	–	–
3	6.75 s	6.82 s	6.77 s	6.73 s	6.50 s	6.88 s	6.67 s
5	7.73 d (9.6)	7.60 d (9.0)	7.61 d (8.5)	7.61 d (8.5)	7.51 d (8.5)	7.62 d (8.5)	–
6	7.23 d (9.6)	6.97 d (9.0)	6.98 d (8.5)	6.99 d (2.0)	6.86 d (8.5)	6.99 d (8.5)	7.24 d (8.5)
7	–	–	–	–	–	–	7.67 dd (8.5)
8	–	–	–	–	–	–	6.97 d (8.5)
2′	7.89 d (9.0)	7.56 d (2.0)	7.43 dd (2.0,2.5)	7.91 d (8.5)	7.38 d (1.5)	8.01 m	7.90 d (8.5)
3′	6.92 d (9.0)	–	–	6.97 d (8.5)	–	7.55 m	6.93 d (8.5)
4′	–	–	7.00 ddd (1.0,2.5,8.0)	–	–	7.54 m	–
5′	6.92 d (9.0)	6.98 d (8.5)	7.39 dd (8.0)	6.97 d (8.5)	6.83 d (8.5)	7.55 m	6.93 d (8.5)
6′	7.89 d (9.0)	7.55 dd (8.5,2.0)	7.48 ddd (1.0,1.2,8.0)	7.91 d (8.5)	7.35 dd (8.5,1.5)	8.01 m	7.90 d (8.5)
5-OMe	–	–	–	–	–	–	3.86 s
7-OMe	3.92 s	3.90 s	–	–	–	–	–
8-OMe	3.91 s	3.96 s	3.95 s	3.95 s	3.93 s	3.93 s	–

Note: Coupling constants (J in Hz) are given in parentheses.

3. Conclusion

Experiments with human liver microsomes had shown that the fully methylated flavanoids resist oxidative metabolism compared to their partially methylated and unmethylated analogues (Walle & Walle, 2007). However, a considerable variation of resistance had been observed among them (Tsuji et al., 2006; Walle & Walle, 2007). Stability of 7-methoxyflavone, for example, was increased or decreased by additional methoxy groups. The variation was from highly susceptible 7,3′-dimethoxyflavone to the more stable 5,7-dimethoxyflavone (Walle & Walle, 2007). The study, which had been conducted with 15 flavones, indicated the importance of positions rather than the number of methoxy groups towards oxidation (Walle & Walle, 2007). This investigation was an attempt to determine the susceptibility of 7,8-dimethoxyflavone towards metabolism and to identify its metabolites prospectively with microorganisms as predictive models for mammalian drug metabolism. The microbial model concept is based on the fundamental grounds that the same Phase I paths of metabolism of xenobiotics observed in mammals are paralleled in microorganisms (Davis, 1987). Of the 40 microbial cultures screened, many were capable of transforming **1** to a limited number of metabolites with each organism. The TLC comparisons of the EtOAc extracts of the culture filtrates showed relatively higher biotransformational efficiency with *M. ramannianus* yielding all metabolites generated by other organisms. The exception was compound **7**, formed with *A. flavus*. About 25% conversion of **1** to the respective metabolites was observed, indicating moderate susceptibility towards oxidative metabolism. It yielded five compounds (**2**–**6**). In compounds **4**–**7**, demethylation occurred at position **7**. The 8-position methoxy group, however, remained unchanged during all the transformations. 5-Methoxyflavone, which was highly resistant to human microsomal oxidation (Walle & Walle, 2007) underwent transformation to metabolites **9** (7.47%) and **10** (71.92%) when fermented with *B. bassiana* and *A. alliaceus*, respectively.

4. Experimental section

4.1. *General experimental procedures*

Unless otherwise stated, the ^{1}H- and ^{13}C-NMR were obtained in DMSO-d_6 on a Varian Unity Inova 600 spectrometer. Optical rotations were determined using a Jasco DIP-370 digital polarimeter. The UV spectra were measured on a Hewlett Packard 8452A diode array spectrometer. IR spectra were run in $CHCl_3$ on an ATI Mattson Genesis series FTIR spectrophotometer. HR-ESI-MS data were obtained using a Bruker GioApex 3.0.

4.2. *Substrates*

7,8-Dimethoxyflavone (**1**) and 5-methoxyflavone (**8**) were purchased from Sigma Aldrich Chemical Co. (Milwaukee, Wisconsin) and their authenticity was confirmed by NMR data.

4.3. *Organisms and metabolism*

Initial screening experiments to select microorganisms capable of converting compounds **1** and **7** to their metabolites in good yields were carried out with 40 microbes from the

collection of the National Center for Natural Products Research of the University of Mississippi. A two-stage procedure was used in all experiments, including the preparative scale fermentations, as described in the literature (Abourashed & Khan, 2000).

Four 2-L flasks, each containing 100 mg of substrate dispersed in 500 mL of medium-α (Herath, Ferreira, & Khan, 2003), were used for preparative scale fermentation of 7,8-dimethoxyflavone (**1**) by *M. ramannianus*. Similarly, 200 mg of **1** in two 2-L flasks were used with *A. flavus*. Six such flasks were used to ferment 250 mg of 5-methoxyflavone (**8**) with *B. bassiana*. The EtOAc extracts of the combined culture filtrates were column chromatographed (silica gel 60 F_{254}) to isolate the metabolites. Repeated column and preparative thin layer (silica gel 60 F_{254}) chromatography were performed to purify the metabolites. Substrate and culture controls were run alongside the above experiments (Jurgens, Hufford, & Clark, 1992).

4.4. *Microbial transformation of 7,8-dimethoxyflavone (1) by* M. ramannianus *(ATCC 9628)*

The ethyl acetate extract of the combined fermentation broth was column chromatographed (Si gel 230–400 mesh: E. Merck, 30 g, column diameter: 20 mm) with CH_2Cl_2 by increasing amounts of MeOH. The fractions were further purified by repeated column and preparative layer chromatography (CH_2Cl_2 : MeOH, 16 : 1) to obtain five compounds (**2**–**6**) that were identified by spectroscopic data.

7,8-Dimethoxy-4′-hydroxyflavone (**2**) was isolated as a white solid (10 mg, 2.5%). R_f 0.42 [MeOH : CH_2Cl_2 (1 : 13)]; UV λ_{max} (MeOH) nm (log ε): 330 (4.31), 306 sh (4.19), 259 (4.16), 251 sh (4.09), 233 sh (4.18), 2.15 sh (4.34), 215 (4.34), 204 (4.42); IR ν_{max}($CHCl_3$) cm^{-1}: 3411, 2939, 1667, 1614, 1512, 1444, 1388, 1290, 1021, 817; ^{1}H-NMR 600 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Tables 1 and 2; HR-ESI-MS $[M + Na]^+$: (m/z) 321.0722 (Calcd for $C_{17}H_{14}O_5 + Na^+$: 321.0738).

3′,4′-Dihydroxy-7,8-dimethoxyflavone (**3**) was obtained as a white solid (30 mg, 7.5%). R_f 0.32 [MeOH : CH_2Cl_2 (1 : 13)]; UV λ_{max} (MeOH) nm (log ε): 340 (3.89), 307 sh (4.70), 2.61 sh (3.71), 241 (3.83), 209 (4.12); IR ν_{max}($CHCl_3$) cm^{-1}: 3444, 2926, 1624, 1582, 1513, 1436, 1377, 1281, 1206, 1033, 810; ^{1}H-NMR 600 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Tables 1 and 2; HR-ESI-MS $[M-H]^+$: (m/z) 313.0750 (Calcd for $C_{17}H_{14}O_6 - H^+$: 313.0721).

7,3′-Dihydroxy-8-methoxyflavone (**4**) was obtained as a white solid (10 mg, 2.5%). R_f 0.26 [MeOH : CH_2Cl_2 (1 : 13)]; UV λ_{max} (MeOH) nm (log ε): 309 (2.70), 284 (2.62), 259 (2.78), 2.39 sh (2.75), 207 (3.04); IR ν_{max}($CHCl_3$) cm^{-1}: 3428, 2921, 1625, 1582, 1448, 1385, 1348, 1203, 1078, 1040, 1004, 959; ^{1}H-NMR 600 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Tables 1 and 2; HR-ESI-MS $[M + H]^+$: (m/z) 285.0745 (Calcd for $C_{16}H_{12}O_5 + H^+$: 285.0764).

7,4′-Dihydroxy-8-methoxyflavone (**5**) was a pale yellow solid (37 mg 9.25%) with a R_f 0.20 [MeOH : CH_2Cl_2 (1 : 13)]; UV λ_{max} (MeOH) nm (log ε): 329 (3.89), 260 (3.73), 2.15 (3.96), 202 (4.09); IR ν_{max}($CHCl_3$) cm^{-1}: 3352, 2952, 1613, 1572, 1510, 1447, 1383, 1293, 1245, 1208, 1079, 826; ^{1}H-NMR 600 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Tables 1 and 2; HR-ESI-MS $[M + H]^+$: (m/z) 285.0779 (Calcd for $C_{16}H_{12}O_5 + H^+$: 285.0764).

8-Methoxy-7,3′,4′-trihydroxyflavone (**6**) was purified as a faint yellow solid (7 mg, 1.75%). R_f 0.10[MeOH : CH_2Cl_2 (1 : 13)]; UV λ_{max} (MeOH) nm (log ε): 330

(4.40), 308 sh (4.31), 260 (4.23), 2.51 sh (4.18), 234 sh (4.27), 214 sh (4.49), 202 (4.66); IR ν_{max} ($CHCl_3$) cm^{-1}: 3121, 2918, 1630, 1507, 1443, 1381, 1290, 1242, 1174, 1076, 824; ^{1}H-NMR 600 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Tables 1 and 2; HR-ESI-MS $[M+H]^+$: (m/z) 301.0850 (Calcd for $C_{16}H_{12}O_6+H^+$: 301.0713).

4.5. *Microbial transformation of 7,8-dimethoxyflavone (1) by* A. flavus *(ATCC 9170)*

The combined culture medium was extracted with EtOAc and the solvent was evaporated. The light brown gummy solid obtained was column chromatographed over silica gel with $CHCl_3$ gradually enriched with MeOH as the eluent.

7-Hydroxy-8-methoxyflavone (**7**) was obtained as a white solid (90 mg, 36%). R_f 0.65 [MeOH : CH_2Cl_2 (1 : 13)]; UV λ_{max} (MeOH) nm ($\log\varepsilon$): 308 (3.34), 259 (3.50), 2.12 (3.62), 201 (3.65); IR ν_{max}($CHCl_3$) cm^{-1}: 3453, 3087, 1635, 1618, 1589, 1568, 1451, 1388, 1363, 1247, 1212, 1073, 1039, 940, 768, 680; ^{1}H-NMR 600 MHz (DMSO-d_6) and ^{13}C-NMR 150 MHz (DMSO-d_6): see Tables 1 and 2; HR-ESI-MS $[M-H]^+$: (m/z) 267.0693 (Calcd for $C_{16}H_{12}O_4-H^+$: 267.0657).

4.6. *Microbial transformation of 5-methoxyflavone (8) by* B. bassiana *(ATCC 7159)*

The EtOAc extract of the combined culture filtrates yielded **9** when column chromatographed over Si gel with CH_2Cl_2 enriched with MeOH.

The white solid, 5-metoxyflavanone (**9**) (65.4 mg, 7.47% yield), showed a R_f 0.85 [MeOH : CH_2Cl_2 (1 : 16)]; $[\alpha]_D^{27}$ 0° ($c=0.51$, MeOH); UV λ_{max} (MeOH) nm ($\log\varepsilon$): 330 (3.87), 269 (4.12), 213 sh (4.49), 202 (4.60); IR ν_{max}($CHCl_3$) cm^{-1}: 2976, 1677, 1601, 1573, 1472, 1103,1090, 786, 734; HR-ESI-MS $[M+H]^+$: (m/z) HR-ESI-MS $[M+H]^+$: (m/z)

Table 2. ^{13}C-NMR data for **2**–**7** and **10**.

	2	**3**	**4**	**5**	**6**	**7**	**10**
C-2	163.4	162.4	162.2	162.7	162.7	162.4	161.0
3	104.8	105.0	106.7	104.6	103.8	107.1	106.8
4	177.2	176.9	177.0	176.8	176.7	177.2	176.8
5	120.8	120.4	120.5	120.4	120.2	120.8	159.5
6	111.2	115.4	116.1	115.4	116.8	115.8	110.4
7	157.0	148.5	151.1	155.7	160.0	155.9	134.5
8	137.0	135.5	135.6	135.5	135.8	135.7	107.6
9	150.4	150.9	151.1	150.9	151.1	151.3	158.0
10	116.6	116.8	116.1	117.1	115.0	117.6	114.1
1′	120.8	122.7	133.2	122.3	121.0	132.1	121.8
2′	128.8	110.3	113.0	128.4	113.0	126.7	128.5
3′	116.8	148.5	158.4	116.5	147.0	129.8	116.4
4′	161.0	150.9	119.1	161.3	150.0	132.2	161.1
5′	116.8	116.4	130.8	116.5	116.6	129.8	116.4
6′	128.8	128.2	117.4	128.4	118.7	126.7	128.5
5-OMe	–	–	–	–	–	–	56.5
7-OMe	61.8	61.3	–	–	–	–	–
8-OMe	57.1	56.3	61.4	61.4	60.9	61.7	–

Note: Measured in DMSO-d_6 at 150 MHz.

255.1109 (Calcd for $C_{16}H_{14}O_3 + H$: 255.10219). Identification of **9** was by comparison with published data (Lee et al., 2007).

4.7. *Microbial transformation of 5-methoxyflavone (8) by* A. alliaceus *(ATCC 10060)*

4′-Hydroxy-5-methoxyflavone (**10**) formed was purified as a white solid by column chromatography (89.9 mg, 71.92% yield). R_f 0.31 [MeOH : CH_2Cl_2 (1 : 16)]; UV λ_{max} (MeOH) nm (log ε): 329 (4.42), 265(4.25), 219 (4.38), 202 (4.45); IR $\nu_{max}(CHCl_3)\,cm^{-1}$: 3428, 2988, 1594, 1580, 1475, 1395,1285, 1131, 834; HR-ESI-MS $[M + H]^+$: (m/z) 269.0811 (Calcd for $C_{16}H_{12}O_4 + H$: 269.08146).

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